



TiO₂ nanoparticle biosynthesis and its physiological effect on mung bean (*Vigna radiata* L.)[☆]

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ABSTRACT

TiO₂ nanoparticle (NPs) biosynthesis is a low cost, ecofriendly approach developed using the fungi *Aspergillus flavus* TFR 7. To determine whether TiO₂ NPs is suitable for nutrient, we conducted a two part study; biosynthesis of TiO₂ NP and evaluates their influence on mung bean. The characterized TiO₂ NPs were foliar sprayed at 10 mgL⁻¹ concentration on the leaves of 14 days old mung bean plants. A significant improvement was observed in shoot length (17.02%), root length (49.6%), root area (43%), root nodule (67.5%), chlorophyll content (46.4%) and total soluble leaf protein (94%) as a result of TiO₂ NPs application. In the rhizosphere microbial population increased by 21.4–48.1% and activity of acid phosphatase (67.3%), alkaline phosphatase (72%), phytase (64%) and dehydrogenase (108.7%) enzyme was observed over control in six weeks old plants owing to application of TiO₂ NPs. A possible mechanism has also been hypothesized for TiO₂ NPs biosynthesis.

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1. Introduction

Titanium is a strong, lustrous, corrosion resistant metal. Its common compound, titanium di-oxide, is a popular photo-catalyst, and is used in the manufacture of pigments [1]. The Ti⁴⁺ ionic state dominate titanium chemistry, owing to its high oxidation state, showing a high degree of covalent bonding. In plants, titanium has been reported to stimulate production of more carbohydrates, encouraging growth and photosynthesis rate [2–4].

TiO₂ is a non-toxic white pigment for use in manufacture of paints, plastics, paper, ink, rubber, textile, cosmetics, leather, and ceramics [5]. Photo catalytic degradation of pesticides with TiO₂ and other catalyst has shown promise as a potential water remediation method [6]. It has also been noted that titanium dioxide breaks down the ethylene gas produced in storage rooms into carbon-dioxide and water, thus it is also used to treat the air in fruit, vegetable, and cut flower storage areas to prevent spoilage and increase the product's shelf life [7].

In the rhizosphere, root exudation is a key process for carbon transfer into the soil, influencing the role of soil microbial communities in the decomposition of organic matter and in native nutrient cycling [8]. Root exudates are the substances released by roots and may affect growth and activity of soil microorganisms in the rhizosphere [9]. Root exudates act as a chemo-attractants to attract microbes toward roots and have been shown to increase the mass and activity of soil microbes [10].

Nanotechnology is one of the most important tools in modern science yet only a few attempts have been made to apply these advances for increasing crop productivity [4,11]. It is possible to develop microorganisms as bionanofactories for synthesis of agriculturally important particles. TiO₂ NPs are promising as efficient nutrient source for plants to increase biomass production due to enhanced metabolic activities, and utilization of native nutrients by promoting microbial activities. Fungi are relatively recent addition to the list of microorganism used in the synthesis of nanoparticles. The use of fungi is potentially exciting since they secrete large amounts of enzymes and are simpler to manage in the laboratory. In the biosynthesis of metal nanoparticles by a fungus, extracellular secreting enzymes are produced which reduce the metal salt of macro or micro scale into nano-scale diameter through catalytic effect. Negative electro kinetic potential of microorganisms enables to attract the cations and act as a trigger for biosynthesis of metal and metal oxide nanoparticles [12,13]. This study attempts to synthesize TiO₂ NPs using *Aspergillus flavus* TFR 7 as an ecofriendly biological approach and evaluate their effect on mung bean (*Vigna radiata* L.).

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Table 1
Characteristics of the experimental soil.

Parameter	Quantity
Sand (%)	83.2 ± 4.2
Slit (%)	6.4 ± 0.8
Clay (%)	6.3 ± 0.5
pH	7.8 ± 0.1
EC (dSm ⁻¹)	0.4 ± 0.04
Organic carbon (%)	0.29 ± 0.01
Total N (mg Kg ⁻¹)	456 ± 13.5
Total P (mg Kg ⁻¹)	719 ± 19.1
Total K (mg Kg ⁻¹)	681 ± 16.8

2. Experimental details

2.1. Characteristic of experimental soil

An experimental soil (20 cm depth) was collected from Jodhpur, India (26°18'N 73°01'E), then air dried and sieved through 2 mm mesh. The soil was classified as loamy sand. Organic carbon was estimated by following the method of Walkley and Black [14]. Nitrogen, phosphorous and potassium were analyzed by Jackson [15]. In addition, pH and electrical conductivity were also measured.

2.2. Isolation and identification of soil fungi

The fungi was isolated from rhizosphere soil by initial plating on Martin Rose Bengal Agar medium (Hi-Media, India, pH 7.2) followed by serial dilutions over potato dextrose agar medium supplemented with chloramphenicol (Sigma–Aldrich, St. Louis, USA) at a concentration of 10 µg mL⁻¹. Isolated fungi was identified up to molecular level by partial sequencing of 18S and 28S rRNA and complete sequence of internal transcribed sequence 1 (ITS-1), ITS-2 and 5.8S rRNA. The sequence was compared with gene library data available on National Centre of Biotechnology Information (www.ncbi.nlm.nih.gov) using nucleotide blast algorithms, to identify isolated fungal strain using bioinformatics tool 'blastn'.

2.3. Synthesis of TiO₂ nanoparticles using soil fungus

To synthesize TiO₂ nanoparticles, *A. flavus* TFR 7 was developed in broth medium (pH 5.8) supplemented with 0.3% malt extract, 1% sucrose, 0.3% yeast extract, and 0.5% peptone. The culture was kept on shaker at 150 rpm at 28 °C for 72 h to develop fungal ball of mycelia. These mycelia were separated out by filtration Whatman filter paper no. 1 (Whatman, UK) followed by triple washing with deionized water. Reaped mycelia (10 g fresh biomass) were re-suspended in 100 mL deionized water and incubated for 48 h

at 28 °C under the same shaking condition as above. The obtained cell free filtrate containing extracellular enzymes was used for synthesis of TiO₂ NPs, in which precursor salt (Bulk TiO₂) was mixed at a concentration of 10⁻³ M and incubated for 36 h at 150 rpm and 28 °C to yield fine monodisperse TiO₂ NPs,

2.4. Characterization of synthesized TiO₂ nanoparticles

Synthesized nano-crystals were characterized morphologically by transmission electron microscopy (TEM; JEOL JEM-2100F) including high resolution (HR)–TEM mode for crystal phase confirmation, and energy dispersive X-ray spectroscopy (EDS; Thermo Noran equipped with TEM) for surface elemental analyses. Since particles were dispersed in water, hydrodynamic diameter was analyzed using dynamic light scattering (DLS; Beckman DelsaNano C, USA).

2.5. Seed germination and exposure of nanoparticles

The certified seed (obtained from institutional seed house) were surface-sterilized using 10% sodium hypochlorite solution followed by triple wash with deionized water. After that, five seeds were sown at 3 cm depth in each pot. The pots were placed in a greenhouse with 16 h photoperiod and 30/20 °C day–night temperature, 60% relative humidity and 360 µmol m⁻² s⁻¹ photo-active radiation intensity. After 10 days of germination, seedlings were thinned to three per pot. The pots were completely randomized and re-positioned weekly to minimize uneven environmental effects.

The experiment was carried out with three treatments viz. control (without TiO₂ application), ordinary TiO₂ (1.6 µ), nano TiO₂ with each of six replicates. The TiO₂ particles (10 ppm) were exposed by foliar application to avoid direct soil contact using a fine nebulizer (25 mL per pot). The concentration and amount of nanoparticle solution was optimized in a preliminary screening experiment (data not shown here).

2.6. Phenological and physiological effect of nanoTiO₂

Plants were harvested after four weeks of foliar application to investigate phenology and physiological state of plant. To analyze, shoots were cut at the soil surface and roots were carefully shaken to remove excess soil, and clumps of soil trapped between roots were removed, and number of nodules, root length, area and diameter were measured using Delta T Scan Software (Delta Scan, UK). To prepare the sample, roots were dipped in a methylene blue dye for 6 h while shoot length was measured on a meter scale.

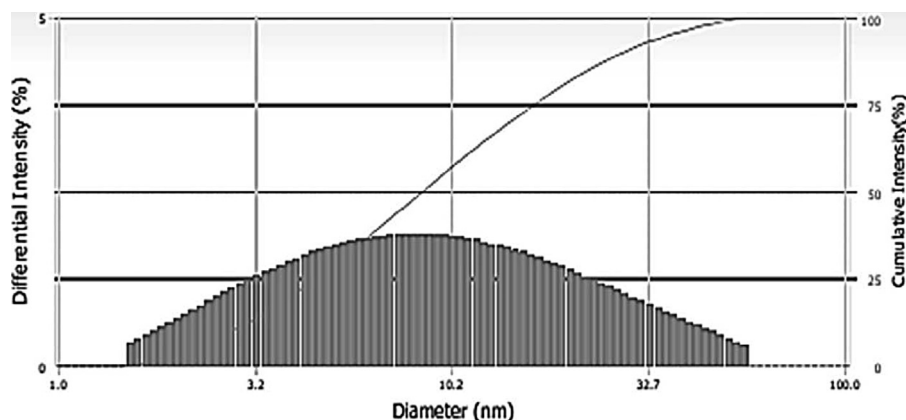


Fig. 1. Size distribution of biologically synthesized TiO₂ nanoparticles.

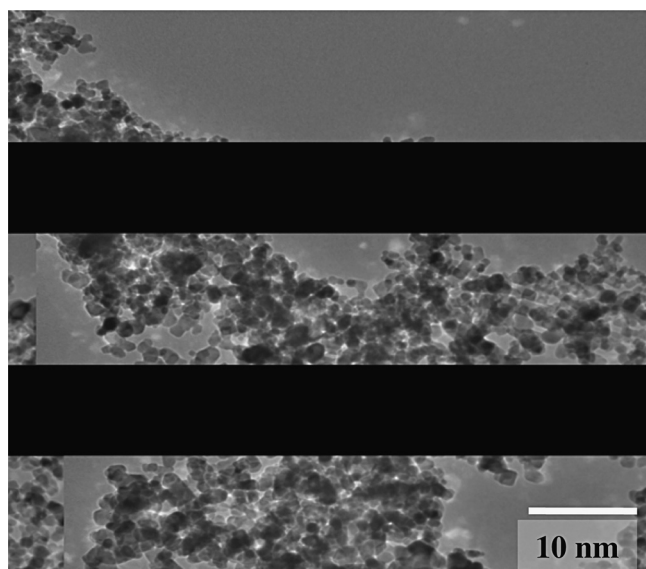


Fig. 2. TEM micrograph of biologically synthesized TiO_2 nanoparticles.

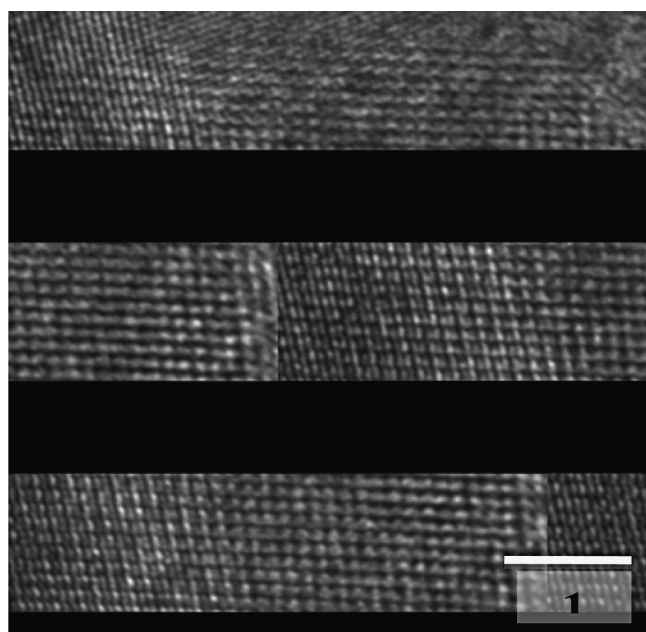


Fig. 3. High Resolution TEM micrograph of biologically synthesized single TiO_2 nanoparticles.

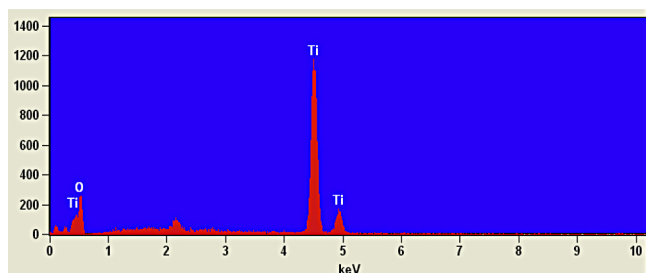


Fig. 4. EDX spectrum of biologically synthesized TiO_2 nanoparticles.

Table 2

Phenological parameter of mung bean plants at six weeks of crop age.

Treatment	Shoot length (cm)	Root length (mm)	Root area (mm^2)	Root nodule
Control	47.0	32.4	16.5	11.7
Ordinary TiO_2	49.6	39.1	19.7	13.3
Nano TiO_2	55.0	48.5	23.6	19.6
LSD ($p = 0.05$)	0.23	0.09	0.07	1.39

Biochemical parameter, dehydrogenase enzyme assay for microbial activity in rhizosphere was assessed according to Tabatabai [16], and phosphorous mobilizing enzymes including acid and alkaline phosphatase activity was assessed according to Tabatabai and Bremner [17]. In addition to these parameter phytase [15], chlorophyll content [18], soluble leaf protein content [14,19] rhizospheric microbial populations were also assayed.

3. Results and discussion

3.1. Physicochemical characteristics of rhizosphere soil

The characteristic of the experimental soils studied are presented in Table 1. The soil was alkaline in nature (pH 7.8) with low electrical conductivity (0.34 dS m^{-1}), organic carbon (0.29%) and NPK contents.

3.2. Isolation and identification of fungi

Isolated fungal strain was identified as *A. flavus* designated with laboratory strain TFR7 on the basis of 5.8S rDNA gene (Complex of -18S-ITS1-5.8S-ITS2-28S) sequence similarity. The gene sequence was submitted to NCBI GenBank and got accession no. of strain, JQ675308 which is available on NCBI the database (<http://www.ncbi.nlm.nih.gov/nucore/383929211>).

3.3. Biosynthesis and characterization of TiO_2 NPs

The biosynthesis of TiO_2 NPs was carried out by exposure of a precursor salt as bulk TiO_2 solution of 10^{-3} M concentration to extracellular enzyme obtained by *A. flavus* TFR 7 in an aqueous solution. The reaction was carried out for 36 h. Synthesized nanoparticles were characterized for morphological analyses.

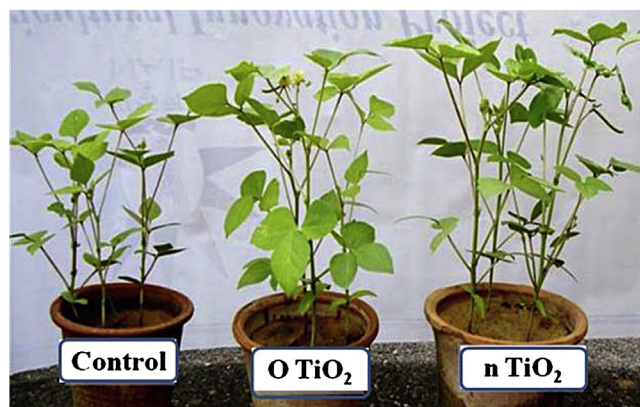


Fig. 5. Phenology of mung bean plant under varying treatment; Control; O TiO_2 : ordinary titanium di oxide; n TiO_2 : nano titanium di oxide.

Table 3

Total soluble protein and chlorophyll content mung bean plants at six weeks of crop age.

Treatment	Total soluble protein (mg Kg ⁻¹)	Chlorophyll content (mg Kg ⁻¹)
Control	8.4	52.3
Ordinary TiO ₂	11.3	60.7
Nano TiO ₂	16.3	76.5
LSD (<i>p</i> = 0.05)	0.18	0.5

Table 4

Microbial population in rhizosphere of six weeks old mung bean plants.

Treatment	Fungi (CFU × 10 ⁻⁴)	Bacteria (CFU × 10 ⁻⁶)	Actinomycetes (CFU × 10 ⁻⁵)
Control	21.0	38.7	13.7
Ordinary TiO ₂	24.0	45.7	15.0
Nano TiO ₂	26.7	47.0	20.3
LSD (<i>p</i> = 0.05)	0.04	0.08	0.05

Table 5

Enzymes activity in rhizosphere of six weeks old mung bean plants.

Treatment	Acid phosphatase (EU × 10 ⁻⁴)	Alkaline phosphatase (EU × 10 ⁻⁴)	Phytase (EU × 10 ⁻²)	Dehydrogenase (pkat g ⁻¹)
Control	5.2	4.3	2.5	5.7
Ordinary TiO ₂	7.4	6.7	3.7	9.5
Nano TiO ₂	8.7	7.4	4.1	11.9
LSD (<i>p</i> = 0.05)	0.09	0.04	0.04	0.14

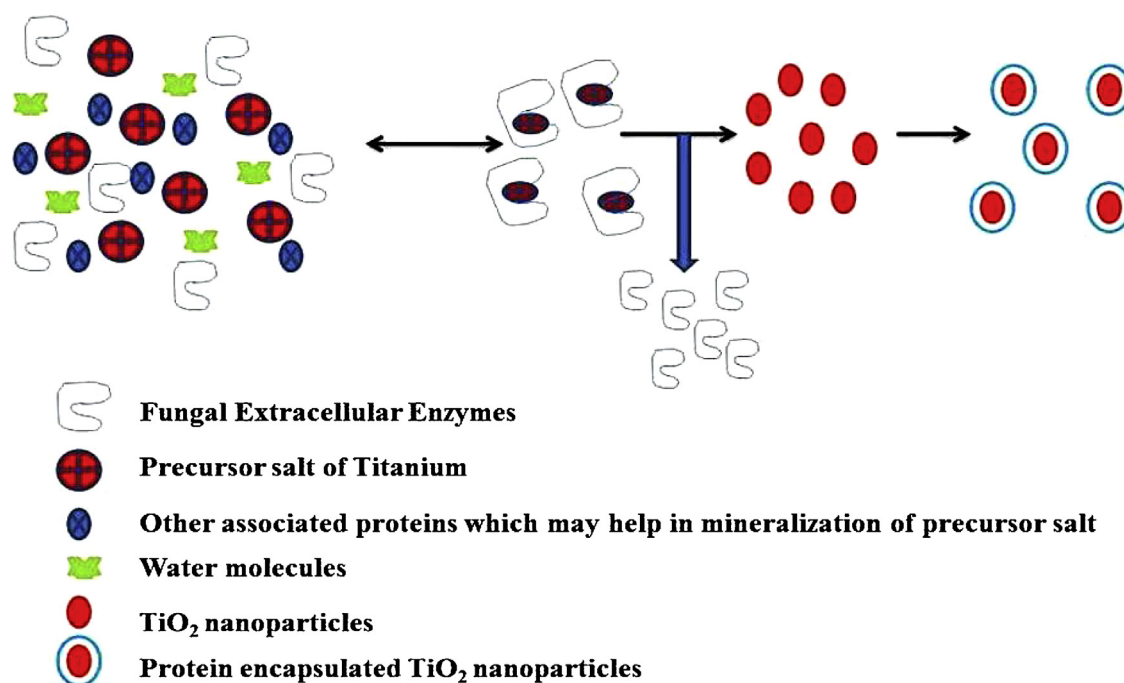
Particle size distribution was analyzed by DLS. Histogram shows average particle size (based on intensity distribution) ranges from 18 nm (Fig. 1). The polydispersity index (PDI) was 0.302 reflects monodisperse nature of the particle. Since DLS measure hydrodynamic diameter, so it was further confirmed with TEM analysis.

TEM measurements showed well distribution of TiO₂ NPs with the average size of 12–15 nm (Fig. 2). Difference in size measurement of TEM and DLS is due to hydrodynamic core that surrounds the particle when dispersed in solvent. The crystal and lattice structure of biosynthesized TiO₂ NPs can be observed in HR-TEM micrograph (Fig. 3). The EDS spectrum (full scan mode) of

drop coated TiO₂ NPs shown in Fig. 4, confirms the purity of titanium metal. The spectrum shows strong peak intensity of titanium metal at 4.6 keV (94 atom%) corresponds to purity of biosynthesized TiO₂ NPs.

3.4. Physiological effect of TiO₂ nanoparticles in mung bean

The results demonstrated a significantly higher plant growth in those plants, which were treated by TiO₂ NPs. With respect to control, plants exposed with TiO₂ NPs showed significant improvements in shoot length (17%), root length (49.6%), root

**Fig. 6.** Mechanism for biosynthesis of TiO₂ nanoparticles.

area (43%) and root nodule (67.5%) due to foliar application of TiO₂ NPs was noticed (Table 2). Clear morphological differences in the phenology of mung bean plant can also be observed in Fig. 5.

Photosynthetic pigment, chlorophyll and total soluble leaf protein content was increased by 46.4% and 94%, respectively (Table 3) due to TiO₂ NPs at 10 mg L⁻¹ concentration. Results of phenology and physiology, clearly indicates that biosynthesized TiO₂ NPs is promising for plant nutrition. Results presented in Table 4, exhibited that population of rhizospheric microbes (fungi, bacteria and actinomycetaceae) was also increased between 21.4% and 48.1% by application at critical growth stage (six weeks) of mung bean crop. Indirectly, TiO₂ NPs also enhance activity of dehydrogenase (108.7%), phytase (64%), acid phosphatase (67.3%) and alkaline phosphatase (72%) in the rhizosphere (Table 5) that may be due to increased microbial population over the control. Increased activity of phytase and phosphatase enzyme activity may help in native phosphorous nutrient mobilization in rhizosphere [20].

Extracellular secretion of enzymes offers the advantage to obtain pure, monodisperse nanoparticles, which are free from cellular components, associated with organisms and easy down-stream processing. Results indicated that *A. TFR 7* is capable to synthesize fine TiO₂ NPs. To understand the mechanism behind biosynthesis of TiO₂ NPs, a simple mechanism is drawn (Fig. 6), showing TiO₂ NPs nanoparticle synthesis using fungus extracellular enzyme secretes. Capping protein, secreted by fungus itself, encapsulates the TiO₂ nanoparticle and increases its stability whereas associated proteins may help in mineralization of precursor salt [21,22]. Detail studies for identification of these proteins and biochemistry investigations are still underway. Such biologically synthesized, functional TiO₂ NPs are economically cheap to synthesize, easy downstream processing and environmentally safe. These promising TiO₂ NPs may act as nanonutrient fertilizer to enhance crop production by stimulating plant metabolic activities. As a nanonutrient, best response of TiO₂ NPs can be perceived by foliar application 10 mg L⁻¹ on 14 days old plant. In plant leaves nanoparticles may adsorb to plant surface and taken up through natural nano or micrometer scale openings. Several pathways exists which are predicted for nanoparticle association and uptake in plants [23,24]. Present invention may open new door for plant nutrition research and fertilizer industries.

4. Conclusion

In the summary, we synthesized an eco-friendly and convenient approach for the synthesis of TiO₂ NPs using *A. flavus* TFR 7 in which no harmful chemical reagent or surfactant template was required, consequently enables the bioprocess with the advantage of being environmental friendly. Synthesized TiO₂ NPs may be used as plant nutrient fertilizer to enhance crop production.

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References

- [1] L. Sang, Y. Zhao, C. Burda, TiO₂ nanoparticles as functional building blocks, *Chem. Rev.* (2014).
- [2] O. Owolade, D. Ogunleti, M. Adenekan, Titanium dioxide affects disease development and yield of edible cowpea, *EJAF Chem.* 7 (2008) 2942–2947.
- [3] M.V. Khodakovskaya, M.H. Lahiani, Nanoparticles and plants: from toxicity to activation of growth, *Handbook of Nanotoxicology, Nanomedicine and Stem Cell Use in Toxicology*, Wiley, 2014, pp. 121–130.
- [4] H. Chen, J.N. Seiber, M. Hotze, ACS select on nanotechnology in food and agriculture: a perspective on implications and applications, *J. Agri. Food Chem.* (2014).
- [5] M. Anpo, P.V. Kamat, *Environmentally Benign Photocatalysts: Applications of Titanium Oxide-based Materials*, Springer, New York, 2010.
- [6] M. Pelaez, N.T. Nolan, S.C. Pillai, M.K. Seery, P. Falaras, A.G. Kontos, P.S. Dunlop, J.W. Hamilton, J.A. Byrne, K. O'Shea, A review on the visible light active titanium dioxide photocatalysts for environmental applications, *Appl. Catal. B: Environ.* 125 (2012) 331–349.
- [7] L.R. Sadler, Apparatus, system, and method for removing ethylene from a gaseous environment, in, Google Patents, 2013.
- [8] E. Baudoin, E. Benizri, A. Guckert, Impact of artificial root exudates on the bacterial community structure in bulk soil and maize rhizosphere, *Soil Biol. Biochem.* 35 (2003) 1183–1192.
- [9] M. SchötteIndreier, U. Falkengren-Grerup, Plant induced alteration in the rhizosphere and the utilisation of soil heterogeneity, *Plant Soil* 209 (1999) 297–309.
- [10] J.L. Butler, M.A. Williams, P.J. Bottomley, D.D. Myrold, Microbial community dynamics associated with rhizosphere carbon flow, *Appl. Environ. Microbiol.* 69 (2003) 6793–6800.
- [11] L.R. Khot, S. Sankaran, J.M. Maja, R. Ehsani, E.W. Schuster, Applications of nanomaterials in agricultural production and crop protection: a review, *Crop Protect.* 35 (2012) 64–70.
- [12] R. Raliya, Appliance of nanoparticles on plant system and associated rhizospheric microflora. PhD Thesis, J. N. Vyas University, Jodhpur, 2012, pp. 199.
- [13] R. Raliya, J.C. Tarafdar, ZnO nanoparticle biosynthesis and its effect on phosphorous-mobilizing enzyme secretion and gum contents in clusterbean (*Cyamopsis tetragonoloba* L.), *Agri. Res.* 2 (2013) 48–57.
- [14] A. Walkley, I.A. Black, An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method, *Soil Sci.* 37 (1934) 29–38.
- [15] M. Jackson, *Soil Chemical Analysis*, Prentice-Hall of India Pvt. Ltd., New Delhi, India, 1967, pp. 41–196.
- [16] M. Tabatabai, Soil enzymes, *Methods of Soil Analysis: Part 2—Microbiological and Biochemical Properties*, Soil Science Society of America, Madison, Wisc, 1994, pp. 775–833.
- [17] M. Tabatabai, J. Bremner, Use of iP-nitrophenyl phosphate for assay of soil phosphatase activity, *Soil Biol. Biochem.* 1 (1969) 301–307.
- [18] H.K. Lichtenthaler, Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, *Methods Enzymol.* 148 (1987) 350–382.
- [19] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, *J. Biol. Chem.* 193 (1951) 265–275.
- [20] A.E. Richardson, R.J. Simpson, Soil microorganisms mediating phosphorus availability update on microbial phosphorus, *Plant Physiol.* 156 (2011) 989–996.
- [21] R. Raliya, J.C. Tarafdar, Biosynthesis and characterization of zinc, magnesium and titanium nanoparticles: an eco-friendly approach, *Int. Nano Lett.* 4 (2014) 1–10.
- [22] R. Raliya, J. Tarafdar, Novel approach for silver nanoparticle synthesis using *Aspergillus terreus* CZR-1: mechanism perspective, *J. Bionanosci.* 6 (2012) 12–16.
- [23] A. Tarafdar, R. Raliya, W.-N. Wang, P. Biswas, J. Tarafdar, Green synthesis of TiO₂ nanoparticle using *Aspergillus tubingensis*, *Adv. Sci. Eng. Med.* 5 (2013) 943–949.
- [24] K.-J. Dietz, S. Herth, Plant nanotoxicology, *Trends Plant Sci.* 16 (2011) 582–589.